Canaigre Investigations

X. FERMENTATION OF CANAIGRE LIQUORS BY YEASTS

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INTRODUCTION

In a previous paper [3] the need for removal of sugars to increase the purity of canaigre extract liquors was pointed out. A method for producing high purity tanning extracts was described in which the sugars are fermented by certain strains of *Aerobacter aerogenes*. This process has been carried out many time in pilot plant size lots of liquor (50 to 300 gal.) and has proved to be satisfactory for producing high purity extracts, except that no easily recoverable, marketable by-products are produced. According to theory [5] 50 per cent of the sugar fermented should appear as nongaseous products. The main non-gaseous product is 2,3-butanediol, which is found in fermented canaigre liquors in concentrations of up to 0.5 per cent [2] or about 65 per cent of theory. Several unsuccessful attempts make it seem very unlikely that this diol could be recovered economically during concentration of the liquors to powdered extracts. A further objection to the method is the formation of insolubles during fermentation [3], consisting of bacterial cells and some amorphous material, which require rather high speed centrifugation for removal.

Obviously it would be advantageous to find some organism that produces both a usable by-product and a high purity extract. To this end we are constantly searching for new types of microorganisms to ferment canaigre liquors. This paper deals with attempts to use yeasts for the fermentation.

MATERIALS AND METHODS

Yeast cultures which we have investigated may be divided roughly into 3 groups on the basis of their reactions to canaigre liquors:

1. Those that are killed or at least fail ro carry out any discernable metabolic process. This group comprises by far the largest number of the cultures tested.
2. Those that do not proliferate but do survive for a limited time and assimilate sugars.
3. Those that multiply and carry out normal metabolic processes.

Most of the work reported here pertains to members of the second group, which have been utilized according to a recently patented process [4]. Since yeasts are known to carry out fermentation processes without reproducing, it was reasoned that massive numbers of yeast cells might be used to remove

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sugars from canaigre liquors and thereby increase the liquor purity, The yeast would first be grown on a suitable medium, then the cells harvested and transferred to canaigre liquor long enough to assimilate the sugar. Recoverable ethanol would be produced in both stages. A search* was made for a yeast which would be comparatively insensitive to the toxic materials in canaigre. Some 60 strains were tested for their activity in increasing concentrations of a canaigre medium. Viability was determined by transfer to agar slants after each exposure. As the concentration of canaigre increased, most of the yeasts died. Eight cultures of group 2 were found which were capable of fermenting sugar in the presence of 6 per cent canaigre extract, equivalent to 3 per cent tannin. In addition, three species representing the third group have been isolated in this laboratory: two from canaigre and one from a sumac liquor. The last is a species of *Torulaspora*, which grows very slowly and destroys tannin and is therefore of no further interest. The first two will be discussed briefly at the end of the next section.

Canaigre liquors were prepared by water extraction of dried shredded canaigre roots. The spent residue remaining after extraction of the tannin always contains a large amount of starch. Since utilization of this material is of great importance in the canaigre development program, acid-hydrolyzed spent canaigre root was investigated as a possible source of nutrients for producing yeast cells. Hydrolysis was accomplished by boiling the ground spent residue under reflux in 2 per cent H_2SO_4 for some of the experiments. However, most of the material was prepared by countercurrent extraction with boiling 6 per cent H_2SO_4, followed by neutralization with calcium carbonate to pH 5–6 and filtration. The latter procedure was preferable for our purpose mainly because it yielded solutions with a higher sugar content. Absolute yield of sugar from the original starch, while of secondary importance in these studies, was close to theoretical.

Both liquors and hydrolyzates were sterilized in 100 to 500 ml. quantities, in flasks with capacities of about 5 times the volume of liquid to be used. All were incubated on a reciprocating shaker at 30° C. for the specified times unless otherwise indicated. Where cyclic fermentation was carried out, passing yeast cells from hydrolyzate to liquor and back again, equal volumes of each substrate were used. Inoculi were obtained by suspending in sterile water the growth from 24–48 hour agar cultures grown at 30° C.

Sugar in the liquors and hydrolyzates was determined by the method of Somogyi [6] after precipitation of the tannin and protein with lead acetate and the excess lead with sodium carbonate. Alcohol was determined on distillates with a dipping refractometer. Tannin analyses of the liquors were made by the official A. L. C. A. methods [1]. Yeast cells were estimated by plating or by direct microscopic counts.

*Dr. L. J. Wickerham, of the Northern Regional Research Laboratory, very kindly made this search among cultures in his collection.

TABLE I

Fermentation of Spent Canaigre Hydrolyzate* by Yeasts.
Incubated 18 hours on a reciprocating shaker at 30° C.

Yeast Culture		Sugar after fermentation %	Sugar fermented % of original	Alcohol gm/100 ml	Alcohol yield % Theoretical	
					Based on original sugar	Based on sugar consumed
NRRL Y 862	Saccharomyces cerevisiae	2.65	73.5	2.92	57.3	77.9
NRRL Y 821	" "	1.27	87.3	3.46	67.8	77.8
NRRL Y 791	" "	1.32	86.8	3.34	65.5	75.4
NRRL Y 813	" "	1.30	87.0	3.50	68.6	78.8
FYC	" "	1.38	86.2	3.31	64.9	75.2
NRRL Y 359	Hansenula anomala	2.46	75.4	2.94	57.6	76.4
NRRL Y 660	Torulopsis utilis	2.57	74.3	2.86	56.1	75.5
NRRL Y 793	" "	2.66	73.4	2.82	55.3	75.4

*Contained 10.00% sugar; no added nutrient.

Results

The eight yeast cultures of group 2 were first tested for their ability to ferment the sugars and to produce alcohol in the hydrolyzates with and without supplemental nutrients. All the cultures grew well with some small differences in the rate of growth and the amount of alcohol produced. Table I shows the results of an experiment with unsupplemented hydrolyzate. After 18 hours' incubation 4 of the cultures had fermented about 87 per cent of the sugar and the other 4 about 74 per cent. Alcohol yields, based on the sugar consumed, were 75 to 79 per cent of theory.* An additional 7 hours permitted all the cultures to consume at least 84 per cent of the sugar, but the maximum was still about 87 per cent. Alcohol yields had not materially increased, and in some cases considerable quantities were lost.

Table II shows the results of fermentation of an hydrolyzate to which various nutrients had been added. The yeast used was Y 813. Potassium phosphate, magnesium sulfate and malt extract had no appreciable effect. Addition of ammonium phosphate or corn steep liquor resulted in the fermentation of slightly more of the sugar and the production of slightly more alcohol than in the unsupplemented control. However, the efficiency of conversion of sugar to alcohol was slightly lower with these two supplements.

*Calculated as 51 per cent of the wt. of sugar.

TABLE II

Fermentation of Spent Canaigre Hydrolyzate* with Added Nutrients by Yeast NRRL Y 813. Incubation 22 hours on a reciprocating shaker at 30° C.

No.	Nutrient Added**	Sugar after fermentation %	Sugar fermented % of original	Alcohol gm/100 ml.	Alcohol yield % Theoretical	
					Based on original sugar	Based on sugar consumed
1	None, inoculated control	2.42	77.9	3.87	69.2	88.8
2	$(NH_4)_2HPO_4$, 0.1%	1.66	84.9	4.01	71.7	84.6
3	K_2HPO_4, 0.05%	2.68	75.5	3.60	64.4	85.3
4	Mg $SO_4.7H_2O$, 0.01%	2.46	77.6	3.75	67.1	86.4
5	Corn steep liquor, 0.1%	1.71	84.4	3.95	70.7	83.7
6	Malt extract, 0.1%	2.57	76.6	3.69	66.0	86.2

* Contained 10.96% sugar.
** Wt./vol. basis.

Further experiments with other nutrient sources led to the tentative conclusion that supplemental nutrients are not necessary, except perhaps a small amount of nitrogen.

TABLE III

Cyclic Fermentation of Spent Canaigre Hydrolyzate* and Canaigre Leach Liquor by Yeast NRRL Y 660, *T. utilis*

Sample No. **	Incubation time hrs.	Sugar after fermentation %	Sugar fermented % of original	Alcohol gm/100 ml.	Alcohol yield % Theoretical		Yeast cells† millions/ml
					Based on original sugar	Based ln sugar consumed	
H 1	24	0.47	83.0	0.92	65.2	78.6	133
L 1	none (control)	1.86	——	——			
L 2	6	0.82	55.9	0.48	50.5	90.6	109
H 2	24	0.43	84.5	0.95	67.4	79.8	156
L 3	16	0.06	96.8	0.77	81.1	83.7	96

*Contained 2.77% sugar.
**H=hydrolyzate; L=liquor.
†Plate count.

Since spent canaigre hydrolzates were found capable of supporting good growth of these yeasts with reasonable yields of alcohol, the next step was to harvest the cells, place them in a canaigre liquor and observe whether the sugars were feremented in the presence of tannin. Table III gives the results of an experiment in which a culture of *T. utilis* was used. It was first grown in an hydrolyzate for 24 hours. The fermented hydrolyzate was then centrifuged and the yeast cells transferred to a canaigre liquor. After 6 hours incubation, the fermented liquor was centrifuged and the yeast cells trans-

ferred to another sample of hydrolyzate. The process was repeated with another sample of liquor which was incubated for 16 hours, thus completing two cycles on each material.

In the hydrolyzates about 84 per cent of the sugar was fermented, and based on the sugar consumed 79 per cent of the theoretical amount of alcohol was produced. In the liquor incubated for only 6 hours, the fermentation was not complete. Only 56 per cent of the sugar had been consumed whereas in the liquor incubated for 16 hours, 97 per cent of the sugar was fermented. Alcohol yields from the liquors were 91 and 84 per cent of theory, respectively. Plate counts indicated that the yeast cells had increased in the hydrolyzates and decreased in the liquors.

TABLE IV

Tannin Analyses of Canaigre Leach Liquors
Fermented by Yeast NRRL Y 660, *T. utilis*

Sample	L 1	L 2	L 3
Incubation Time, hrs.	none	6	16
Soluble Solids %	5.41	4.45	3.61
Nontannin %	2.69	1.65	0.98
Tannin %	2.72	2.80	2.63
Purity 100T/SS	50.3	62.9	72.9
Total Sugars%	1.86	0.82	0.06

Table IV shows the tannin analyses of the fermented liquors. In the liquor fermented for 6 hours the purity (100 x Tannin/Soluble Solids) increased from 50 to 63 and in the 16 hour fermented liquor to 73. Since the analyses were run on centrifuged liquors there were no insolubles present. Tannin values did not change significantly.

An experiment in which an hydrolyzate containing 8.2 per cent sugar and a liquor containing only 0.8 per cent sugar were fermented by yeast NRRL Y 821 is reported in Table V. The hydrolyzate was inoculated with 3 million yeast cells per ml. After 19 hours' incubation there were 170 million cells per ml. by direct microscopic count and 41.5 per cent of the sugar had been consumed. After an additional 2 hours the cell count was 200 million/ml. and 57 per cent of the sugar had been fermented. At this point the cells were harvested by centrifugation and placed in the liquor, giving a cell count of 180 million per ml. After 5 hours 50 per cent of the sugar had been consumed; the cells were so badly clumped that they could not be counted. After an additional 20 hours, 75 per cent of the sugar had been consumed, the tannin content had not changed and the purity increased from 55 to 63. The harvested cells, incubated for 19 hours in a second aliquot of the hydrolyzate, increased to 450 million / ml. and consumed 79 per cent of the sugar. In a

TABLE V

Fermentation of Spent Canaigre Hydrolyzate* and Canaigre Liquor** by Yeast NRRL Y 821

Sample No.***	Incubation time hrs.	Sugar fermented % of original	Soluble solids %	Tannin %	Purity 100T/SS	Yeast cells† X 10⁶
H 1	0	——				3
H 2	19	41.5				170
H 3	21	57.3				200
L 1	0	——	3.19	1.74	54.5	180
L 2	5	50.0				‡
L 4	25	75.0	2.63	1.65	62.7	‡
H 5	19	79.3				450
L 6	6	62.5				‡
L 7	22	62.5	2.55	1.61	63.1	‡

* Contained 8.2% sugar.
** Contained 0.8% sugar.
*** H=hydrolyzate; L=liquor.
† Direct microscopic count per ml.
‡ Could not count, cells clumped.

second aliquot of liquor, these cells consumed in 6 hours almost $^2/_3$ of the sugar, which amount was not increased by additional incubation. Alcohol yields in this experiment were of the same order of magnitude as those reported in the previous experiment.

Considerable attention has been given a new species of *Endomycopsis*,* a member of the third group of yeasts described in the preceding section. It has been isolated repeatedly from various canaigre preparations in which it grows very well. Unfortunately, it destroys tannin. Table VI shows a typical result when this yeast was used to ferment canaigre liquor. In this particular experiment there was a 12 per cent tannin loss. Many attempts, all unsuccessful, have been made to induce this organism to destroy the sugars in canaigre liquors without destroying tannin.

*Identified by Dr. L. J. Wickerham.

TABLE VI

Fermentation of Canaigre Liquor with *Endomycopsis sp.* 24 hrs. incubation with aeration at 30° C.

	Control	Fermented
Soluble Solids %	4.90	3.55
Insolubles %	0.24	1.08
Nontannin %	2.26	1.23
Tannin %	2.64	2.32
Purity Tan / SS X 100	53.9	65.4
Total Sugar %	1.1	0.06

TABLE VII

Fermentation of Canaigre Liquor with an Unidentified Yeast, Y 89
14 hrs. incubation with aeration at 30° C.

	Control	Fermented
Soluble Solids %	3.19	2.37
Insolubles %	0.02	0.04
Nontannin %	1.45	0.67
Tannin %	1.74	1.70
Purity Tan / SS X 100	54.6	71.7
Total Sugar %	0.8	0.06

The final member of the third group was isolated from a solution of canaigre liquor containing about 10 per cent of a synthetic tannin, Orotan,** resulting from an extraction experiment. After standing in the laboratory for several weeks this solution formed a light-colored sediment, which was sub-cultured to yield a yeast designated as Y 89. This culture has not yet been completely identified. It is probably a haploid form of a species closely related to, but not identical with *Hansenula anomala.* As shown in Table VII, canaigre liquor can be fermented by this culture without loss of tannin.

Further studies will be made with this organism since at present it looks very promising.

Discussion

On the basis of these and other tests, a plan shown in Figure 1 has been drawn up for utilizing the cycling process in a canaigre processing plant. Lack of suitable equipment has prevented our actually carrying out the complete process. It should be pointed out that our experimental conditions, e. g., incubation on a shaker, were not designed for maximum alcohol yield and, therefore, would hardly be retained in a plant process. No doubt the alcohol yields from hydrolyzates could be improved by anaerobic fermentation with mechanical agitation, although it is uncertain whether this would produce sufficient yeast cells for fermenting the liquors. Also there was little attempt made to determine the best method for hydrolysis of the residue from a cost and efficiency standpoint. Such studies would obviously be required for evaluation of the process as a commercial venture.

The spent residue from the extraction unit would be hydrolyzed by acid or enzymes. After any necessary neutralization, filtration, etc., the sugar-containing solution would be fermented continuously. The fermented hydrolyzate would be run through a continuous centrifuge or filter, the yeast cells going to the liquor fermenter and the beer to stills for recovery of the alcohol. The liquor fermenter would also run continously. The fermented liquor

**Mention of specific products in this paper is not to be construed as a recommendation or endorsement by the Department of Agriculture over similar products not mentioned.

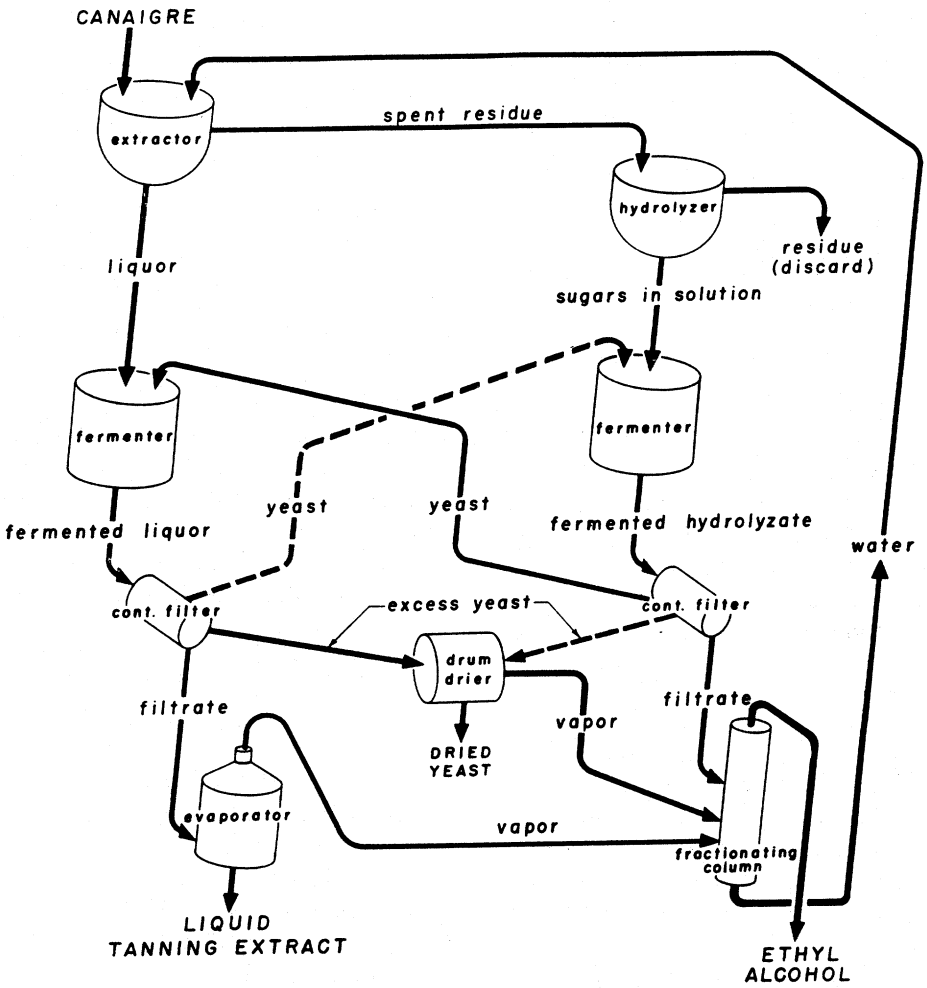

FIGURE 1.—Plan for utilizing cyclic yeast fermentation process in a canaigre extract plant.

would be centrifuged or filtered, the liquor going to an evaporator for production of liquid tanning extract and recovery of alcohol. The yeast cells, if needed, would be recycled to the hydrolyzate fermenter; any excess would be dried for feed as an additional by-product. If properly balanced, there appears to be no technological reason why this process should not be successful after the details have been worked out.

It would seem that enough alcohol might be produced to make its recovery economically feasible. It has been estimated * that a daily production of at

*Personal communication from P. Burke Jacobs, Industrial Specialist (alcohol) Northern Regional Research Laboratory, Peoria, Illinois.

least 2,000 gallons might be necessary in order to carry the essential plant labor and technological control without incurring too high an overhead charge per gallon of alcohol.

On the basis of figures which are admittedly rather tenuous, the production of 2,000 gallons of 95 per cent alcohol from the liquors and spent residues would require that from 90 to 100 tons of fresh roots be processed per day, yielding about 8–10 tons of dry extract containing 60 per cent tannin. Daily production of 10 tons of dry extract would amount to about 1,500 tons of 100 per cent tannin per year, based on 250 working days. This would constitute about 1¼ per cent of the total current domestic consumption of vegetable tannin, of which about 85 per cent is now being imported. If competitive price and quality of the extract were favorable, it is obvious that the market would readily support a number of plants such as the one proposed.

Summary

The action of various yeasts on canaigre liquors is described. Most of the yeasts tested were killed by the liquor. A few cultures were found which can tolerate the liquor and assimilate the sugars but they do not grow nor proliferate. Members of this group have been utilized in a process in which the yeast is first grown in a suitable medium such as hydrolyzed spent canaigre. The cells are then harvested and placed in the liquor to be fermented. After the sugars have been fermented the cycle is repeated. Alcohol may be recovered from both media. A plan is given for utilizing this process on a continuous basis.

A third group of yeasts consists of those that multiply in canaigre liquors and carry out normal metabolic processes. Three such species have been isolated. Two of these destroy tannin, but one can be grown in canaigre liquors without attacking tannin and is being investigated.

Acknowledgments

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